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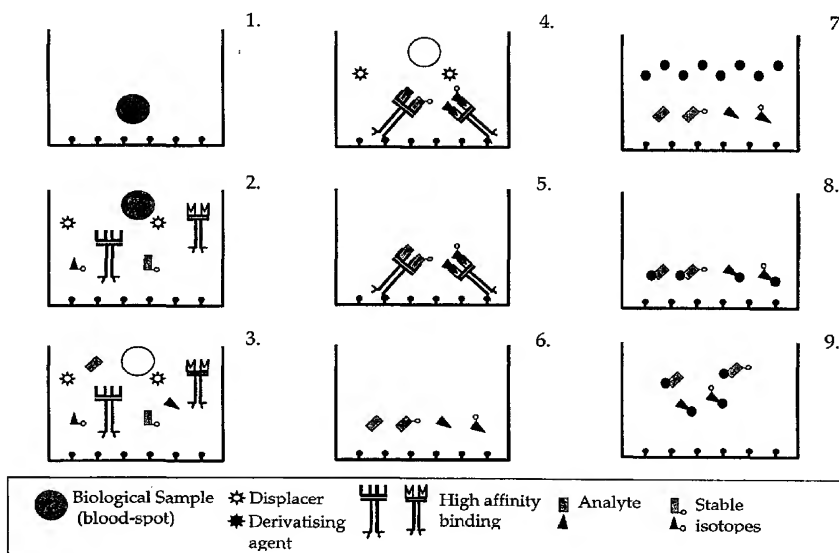
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(54) Title: DETECTION OF COMPOUNDS SUCH AS THYROXINE

A diagrammatic representation of the steps in the SMAC-MS process



(57) Abstract: A novel method, referred to as Simultaneous Multi-Analyte Capture Mass Spectrometry (SMAC-MS), is disclosed for the quantification of analytes, in particular thyroxine (T₄) and 17- α -hydroxyprogesterone (17OHP) in dried blood spots of newborns for the purposes of mass screening and diagnosis of congenital hypothyroidism (CHT) and congenital adrenal hyperplasia (CAH). The method enables a high-throughput and automated process that will be fully integrated into neonatal screening programmes. The method may also be provided by a test kit to carry out the process.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DETECTION OF COMPOUNDS SUCH AS THYROXINE

The present invention relates generally to methods for quantifying levels of thyroxine (T_4) and 17- α -hydroxyprogesterone (17OHP), or similar, low molecular weight compounds, in samples taken from humans and other mammals. In particular, this invention allows high throughput mass scale detection by simultaneous quantification of the levels of T_4 and 17OHP or related compounds in blood samples by affinity capture of T_4 and 17OHP followed by chemical derivatisation and quantification using stable isotope dilution tandem mass spectroscopy (MS/MS) for the purposes of accurately diagnosing and screening newborn infants for Congenital Hypothyroidism (CHT), Congenital Adrenal Hyperplasia (CAH) and related congenital disorders.

The term low molecular weight compounds or LMW compounds is taken to refer to compounds with a mass to charge ratio (m/z) of up to 2000.

The term analyte is taken to refer to the substance being measured in an analytical procedure.

BACKGROUND

Newborn screening is a preventative public health program that is performed, in most developed countries, for the early detection of congenital disorders. The purpose of newborn screening is to screen all newborns in order to identify, diagnose and treat specific disorders which, if not treated, will cause preventable irreversible harm to the infant. The newborn screening test is routinely done on filter paper blood specimens (dried blood-spots) collected from neonates in the first few days of life. The first dried blood-spot screening test was implemented by Robert Guthrie in 1962 for the identification of infants with Phenylketonuria (PKU). This screening test became known as the Guthrie Test and became the first population-based screening program for a genetic disorder. Neonatal screening test for CHT was introduced in

the early 1970's, that was later followed by screening for CAH. More recently, with the introduction of MS/MS into newborn screening programs, the number of genetic disorders screened for in newborns has been greatly expanded.

Newborn screening for CHT is undertaken on a wide scale and is currently performed by using a number of different strategies: the measurement of Thyroid Stimulating Hormone (TSH) alone, both T_4 and TSH, or T_4 initially as the primary screen and the subsequent measurement of TSH on a certain percentage of the lowest T_4 values of the population. Both the measurements of T_4 and TSH are performed using discrete immunoassay techniques from dried blood-spot samples. The most comprehensive screening for CHT would be achieved by simultaneous measurements of T_4 and TSH. This strategy would allow for the identification of both primary and secondary CHT, in addition to significantly reducing the false positive rate. The most widely used neonatal CHT screening protocol uses the measurement of TSH alone to identify infants with CHT. Some neonatal screening protocols measure primary T_4 , followed by confirmation by measuring TSH. These CHT screening strategies have limitations either of only detecting primary hypothyroidism or having a large proportion of false positive results.

Therefore, the preferred screening method for CHT is the simultaneous measurement of T_4 and TSH analytes on each filter paper blood-spot specimen, as that ensures higher detection rates and lower recall rates and false positive results.

The measurement of both the analytes in this manner is problematic in that it is a time-consuming, expensive and technically involved process that is not used for the purposes of mass newborn population screening.

As part of the prior art, De Brabandere *et al* disclose the concept of the quantification of T_4 using mass spectroscopy. However, when this technique is used directly on extracts from dried blood- spot samples taken from infants, the results are insensitive

and are characterized by high levels of background noise making it difficult to accurately diagnose infants with CHT. The concept of trapping an analyte by an antibody and subsequent analysis using mass spectroscopy has previously been described by Nelson *et al.*

A prior art method of detection described by Krone *et al* is by Matrix-Assisted Laser Desorption Ionisation (MALDI), but this apparatus/technique does not allow for mass screening. Therefore, although techniques for diagnosis of CHT by measurement of T₄ by MS/MS have been reported, they are either insufficiently accurate, insensitive or unsuited for mass newborn screening.

CAH, a disorder caused by a deficiency of the 21-hydroxylase enzyme, is the most common genetic disorder of the adrenal steroid pathway. The newborn screening for CAH is currently performed by measuring 17OHP from dried blood-spots using immunoassay techniques. The problem with current immunoassay based methods is that they are affected by a significant degree of interference as a result of cross-reactivity of the 17OHP antibodies with other steroids. This is particularly a problem in sick and premature neonates and results in high recall rates and false positive results. This can be overcome by using MS/MS as it affords high sensitivity, specificity and accuracy in the quantification of 17OHP that cannot be achieved by immunoassay techniques.

It is the objective of this invention to enable the specific determination and quantification of the concentration of LMW compounds such as T₄ and 17OHP that are present in biological samples (dried blood-spots) at concentrations usually below 1 μ M.

SUMMARY OF THE INVENTION

The following invention comprises a series of procedures that alleviate the aforementioned problems by providing a sensitive technique to more accurately

detect quantities of an analyte. Although newborn screening using T₄ for the diagnosis of CHT and 17OHP for the diagnosis of CAH is described as the preferred embodiment, similar methods can be used to isolate other compounds from interfering substances in biological samples, such as blood spots, for the detection of various disorders, conditions or diseases.

According to one aspect of the invention, there is provided a method of measuring the quantity of an analyte in a biological sample taken from a mammal, including the steps of:

- (a) preparing at least one biological sample, in which said analyte is present, from said mammal;
- (b) capturing said analyte from said sample, using an agent having affinity for said analyte;
- (c) coupling one or more detectable moieties to said analyte; and
- (d) quantifying the levels of said analyte by mass spectrometry scanning.

In the preferred form, the said analyte has a mass to charge ratio (m/z) of up to 2000.

In the alternative, the said analyte is selected from the compounds T₄ or 17OHP, or a derivative thereof.

In a second alternative the said T₄ is present in said biological sample at a concentration of up to 1 μ M.

In the preferred form the said agent having affinity for said analyte is selected from the group consisting of globulin proteins, monoclonal or polyclonal antibodies, sera, aptomers and antibody or receptor fragments; or derivatives thereof.

In the preferred form, the said method is applied to the mass screening of newborns.

In the alternative the said method for screening for congenital hypothyroidism or congenital adrenal hyperplasia.

In the preferred form the said detectable moiety is in the form of an acyl ester.

In the preferred form the said detectable moiety is in the form of a butyl ester coupled to T₄.

In a second aspect of the invention a test kit for the detection and/or determination of the concentration of an analyte includes:

- (a) microtitre plates containing wells that are coated with avidin
- (b) additional immuno chemical reagents including:
 - (i) an elution buffer containing a displacer of salicylate
 - (ii) an aqueous buffer containing agent(s) coupled with biotin molecules and having affinity for said analyte
 - (iii) a solution having stable isotopes of T₄ and 170HP
 - (iv) a solution having a chemical derivatisation reagent
 - (v) a washing buffer
 - (vi) a solvent delivery buffer

In the preferred form, said agent(s) having affinity for said analyte are coupled with biotin molecules, the said agent(s) in the form of one or more of the following; monoclonal or polyclonal antibodies, globulin proteins, sera, aptomers and antibody or receptor fragments or derivatives thereof.

In the preferred form, said chemical derivatisation agent results in the butylation of the said analyte, such as T₄ or 17OHP.

Hence there is provided according to this invention a method of selectively capturing the LMW compounds with high affinity-binding agents followed by the selective derivatisation and quantification using MS/MS. This process enables the selective

removal of non-specific, non-binding biological compounds that are responsible for the problem of signal suppression encountered when LMW compounds are analysed by MS/MS. Overcoming this signal suppression problem imparts an increase in the signal to noise intensity ratio allowing for the quantification of LMW compounds with greater accuracy and precision from biological samples such as a dried blood-spot. This process will vastly improve methods of screening newborns by enabling a cost-effective rapid sample throughput system using a MS/MS method. The full potential of MS/MS with its high sensitivity and specificity can be applied to the detection of a broad spectrum of LMW compounds using the MS/MS system for the newborn screening of congenital genetic disorders.

It will be seen therefore that the present invention relates to a high throughput newborn screening method that is cost-effective with high sensitivity and specificity for the accurate determination of LMW compounds such as T₄ at concentrations normally present at less than 1 μ M in biological samples. The process involves the simultaneous selective capture of LMW compounds using high affinity binding agents, such as binding proteins, antibodies, aptomers or receptors with the subsequent removal of non-specific, non-binding compounds followed by derivatisation of the captured LMW compounds and their quantification using MS/MS.

This process virtually overcomes the problems of signal suppression encountered when determining and quantifying of LMW compounds particularly at concentrations below 1 μ M from biological samples such as dried blood-spots. The process can be applied to mass newborn screening for the purpose of identifying individuals determined to be at increased risk as a result of the concentration of T₄ that is below or above a population threshold resulting in the need of medical intervention and further specialised diagnostic testing.

Even when only the smallest quantities of samples are available for testing, the high sensitivity resulting from derivatisation or chemical modification ensures accurate

detection of the concentration of T_4 for the identification and diagnosis of infants with CHT.

DETAILED DESCRIPTION OF THE INVENTION

The following is a description of the preferred technique to be used for quantifying T_4 and 17OHP levels accurately by minimizing signal suppression and maximizing the response of the signal to noise ratio required for detection. Although T_4 and 17OHP are used as examples, this technique can be used to isolate other analytes.

For the purpose of the demonstration of the process MS/MS analyses were performed using a Perkin-Elmer SCIEX (Concord, Ontario, Canada) API 365 triple quadrupole mass spectrometer with an ionspray source and a syringe pump (Harvard Apparatus, Cambridge, MA, USA) or autosampler (Gilson, France) for infusion into the MS/MS electrospray source via a fused silica transfer line at a flow rate of 5 μ L/min.

Fig 1 is a diagrammatic representation according to the invention for measuring the concentration of analytes such as T_4 and 17OHP from a biological sample such as a blood-spot. It would be known to a person skilled in the art that many variations exist.

Fig 2 is a mass spectral scan of T_4 at a concentration of 345 μ M from a 3mm filter paper spot collected onto Schleicher and Schuel 903 filter paper. Chromatogram (A) contains the T_4 standard only and chromatogram (B) contains the T_4 standard in the presence of whole blood. In the presence of whole blood the signal intensity of T_4 was reduced by 85%. The experiment performed was a Multiple Reaction Mode (MRM) monitoring on the ion pair of m/z 834/732.

Fig 3 show the results for the determination of the concentration of T_4 from human serum.

Fig 4 shows that the determination of T_4 and 17OHP can be performed simultaneously within the same reaction tube or microtitre well using a single blood-spot specimen. The determination of 17OHP by MS/MS is unaffected by the prior treatment with butanolic-HCL during the butylation of T_4 . In Fig 4A the intensities of the products of the molecular ion of 17OHP at m/z of 97.1 and 109.1 are unaffected by chemical treatment with butanolic-HCl at a concentration of 50nM. Fig 4 B shows the signal intensity of the molecular ion of T_4 from the neutral total loss of m/z 102 performed at concentration of 500nM.

Referring to Fig 1, there are nine main experimental steps involved in the embodiment that are important to allow rapid, automated, sensitive and accurate quantification of the analytes from a biological sample.

In step one, the biological sample, a dried blood-spot is placed by an automated punching machine into a reaction vessel, such as a microtitre well that has been pre-coated with a binding agent.

In steps two and three, the additives of high affinity binding agents such as monoclonal antibodies, displacer agent, such as salicylate and stable isotope internal standards are added to the reaction vessel within an aqueous buffered solution using an automated sample delivery equipment.

In step four, after a reaction period, the analytes that have been eluted from the blood-spot and the internal standards have bound to their respective high affinity binding agents. The monoclonal antibodies have also been bound to the binding agent that is adhered to the walls of the reaction vessel.

In steps five and six, the reaction vessel is washed with an aqueous solution using an automated washing equipment to remove all material that is not bound to the high

affinity monoclonal antibodies and followed by the addition of an organic solvent such as methanol to solubilise the bound analytes and internal standards.

In steps seven and eight, the methanol is removed by evaporation under a stream of nitrogen and derivatising agent or agents such as butanolic-HCl is added to chemically modify selected analytes, such as T₄, once the reaction is complete the derivatising agent is again removed by evaporation under a stream of nitrogen.

In step nine, a solvent to aqueous solution is added such as acetonitrile, water and formic acid in the ratio of 50:50:0.025 by volume to each dried reaction vessel and the samples are then loaded into the MS/MS, which performs automated analysis procedures with speed and reproducibility.

Methods and Results:

In this experiment the reaction was performed by the addition of 10 µL of human serum containing T₄ at zero (Fig 3A) or 300 nmol/L (Fig 3B) to which was added 30 µL of a buffer solution containing a displacer and 10 µL of affinity purified monoclonal antibody to T₄ at a concentration of 150 nanograms in a final volume of 50 µL. This mixture was incubated at 4 degrees Celsius for 4 hours allowing the antibody to bind to the added T₄. For Fig 3C no affinity purified monoclonal antibody was added to the reaction mixture made up of human serum and spiked with 10 µL of a 1 µM/L T₄ solution.

At the end of the reaction 50µL of Protein-A sepharose slurry was added to all three reaction tubes and incubated with mixing for 2 hours at room temperature. The reaction vials were centrifuged at 13000 rpm and the supernatant removed leaving the pellet. The pellet was washed with 1 milliliter of phosphate buffered saline buffer at pH 7.8 and the pellet re-suspended and centrifuged at 13000 rpm. This was repeated twice more with the final wash being done with a solution of ammonium

bicarbonate. The pellet from the last wash was lyophilized to remove the remainder of the wash buffer.

To the dried pellet was added methanol containing the stable isotope of $^2\text{H}_5$ -phenylalanine and this was mixed for 15 minutes at room temperature and centrifuged at 13000 rpm.

This could also be achieved by covalently coupling to biotin molecules to high affinity antibodies or binding protein, in the presence of a displacer (examples: borate salt or salicylate), which frees T_4 from blood proteins (eg albumin, thyroid binding protein), where it can then bind the modified high affinity antibodies or binding proteins. The biotin antibody complex is then bound to avidin that is anchored to a solid support.

The supernatant was removed and placed into a polypropylene tube and the methanol evaporated off under a stream of nitrogen. To the dry tubes was added 50 μL of butanolic-HCl and reacted at 65 degrees Celsius for 15 minutes. The butanolic-HCl was evaporated under a stream of nitrogen and to each dry tube was added a solution of acetonitrile: water: formic acid at a ratio of 50:50:0.025 volume. The solutions can then be analysed using MS/MS.

Fig 3 demonstrates that the use of a monoclonal antibody to capture T_4 and subsequently removing all other signal suppressing agents is capable of determining T_4 at levels below $1\mu\text{M}$ from a biological sample such as serum (Fig 3B). The specificity of the process is shown in Fig 3C where in the absence of the monoclonal antibody no signal at m/z 834 could be identified even at a concentration of T_4 at $1.3\mu\text{M}$.

Fig 3A shows that there is no signal at m/z 834 when a serum sample containing no T_4 was used in the process. Quantification is made possible by the inclusion of a

stable isotope of T_4 at the time of elution from the biological matrix, thus enabling both endogenous and isotope-labelled T_4 to be captured by the solid phase antibody. For each of the experiments direct comparison was made relative to the intensity of the molecular ion at m/z 227 representing the butylated ester of 2H_5 -phenylalanine determined by neutral total loss of m/z 102 scan.

Discussion:

When analyzing complexes of T_4 in biological matrix, MS/MS signal suppression is of major concern and prevents accurate identification and quantification to enable diagnosis. Immunoaffinity capture of T_4 can be used to significantly reduce signal suppression. T_4 can be isolated from associated biological matrix, such as a dried blood-spot, and other signal suppressing compounds by using, for example, a reagent comprising high affinity antibodies or binding protein that are either in solution, bound to a solid support or have been chemically modified by coupling to another compound.

DERIVATISATION OF CAPTURED T_4 :

On average, a 3mm dried blood spot is taken from neonates for analysis. After immunoaffinity capture of T_4 and quantification using MS/MS, the high level of background noise levels can still affect the ability to accurately determine the concentration of T_4 . The signal to noise ratios can be further increased by the derivatisation of T_4 . In particular, the formation of an acyl ester, such as a butyl-ester (using butanolic-HCL) of T_4 and monitoring of the molecular ions 834/732 results in a significant 10-fold increase in the signal to noise ratio. This particular modification significantly increases the accuracy and precision of quantification of the concentration of T_4 by MS/MS.

Table 1. Intensities obtained from the precursor ion scan of the product ion at m/z 732 of the butyl ester of T_4 and underivatised T_4 at a concentration of $3.5\mu M$.

Sample	MS/MS Intensities	Q1 Scan Intensities
Underivatised T ₄	1.8 × e*6	2.7 × e*7
Butyl ester of T ₄	2.0 × e*7	2.0 × e*8

In Table 1 a comparison is provided showing the signal intensities of T₄ by MS/MS with and without derivatisation T₄. There is an increase in signal intensity associated with the formation of the butyl-ester of T₄ over the underivatised T₄. This increase in signal intensity enables the determination of T₄ to a concentration of 15nM, which is 10 times above the signal to noise ratio. Optimisation of the MS/MS had been performed for both the T₄ butyl ester and underivatised native T₄.

In addition, when performed in the absence of biological matrix, which is removed by the previous capture step, this technique enables quantification of T₄ to concentrations of less than 50 nmol/L. The published cut-off level for T₄ required for neonatal CHT screening is approximately 100 nmol/L whole blood (Dussault *et al*) and/or the use of the lowest 10% of T₄ population.

Current techniques utilising MS/MS in infant screening for inborn errors of metabolism by determination of amino acids and acyl carnitines from blood spots include a derivatisation step for the formation of butyl esters. The chemical derivatisation by formation of the butyl ester can be conveniently adapted into existing newborn screening blood-spot protocols for large scale processing.

In addition, the chemical derivatisation of the primary amino group or carboxyl group of the T₄ molecule by formation of a quaternary ammonium analogue or an acyl ester, respectively, provides greater increases in signal sensitivity and thus greater sensitivity. The quaternary moiety provides a permanent positive charge on

the T₄ molecule, thus removing the need for ionisation at the inlet source of the MS/MS.

Furthermore, the process of derivatisation by formation of a butyl ester using butanolic-HCl does not interfere with the measurement of 17OHP by MS/MS. This process enables the simultaneous measurement of T₄ and 17OHP from a single blood-spot sample performed within a single reaction well.

DETECTION USING MS/MS:

The use of MS/MS in frontline newborn screening for the detection of inborn errors of metabolism is rapidly increasing world-wide. This invention brings CHT and CAH screening into line with other infant screening testing procedures that utilise MS/MS. The existing limitations of determining T₄ and 17OHP by mass spectrometry suitable for mass screening are overcome by specific capture followed by chemical derivatisation to increase MS/MS signal to noise ratio. The ability to automate the system for MS/MS is a major advantage.

The invention allows the determination of LMW compounds even at concentrations below 1 μ M using MS/MS. In particular, the signal suppression is significantly reduced. The signal suppression is mainly due to lipids, salts and blood pigments present in blood-spots, and these constituents can reduce the signal intensity of a single species to about 1-5%.

Whilst the published methods use high pressure liquid chromatography MS/MS to overcome this problem they are inadequate in that they do not allow for the high throughput necessary for a neonatal screening test. This system can be applied to the mass screening of CHT and CAH either as first-tier or second-tier approaches in identifying, clarifying or for further testing of dried blood-spots for the concentration of indicative analytes.

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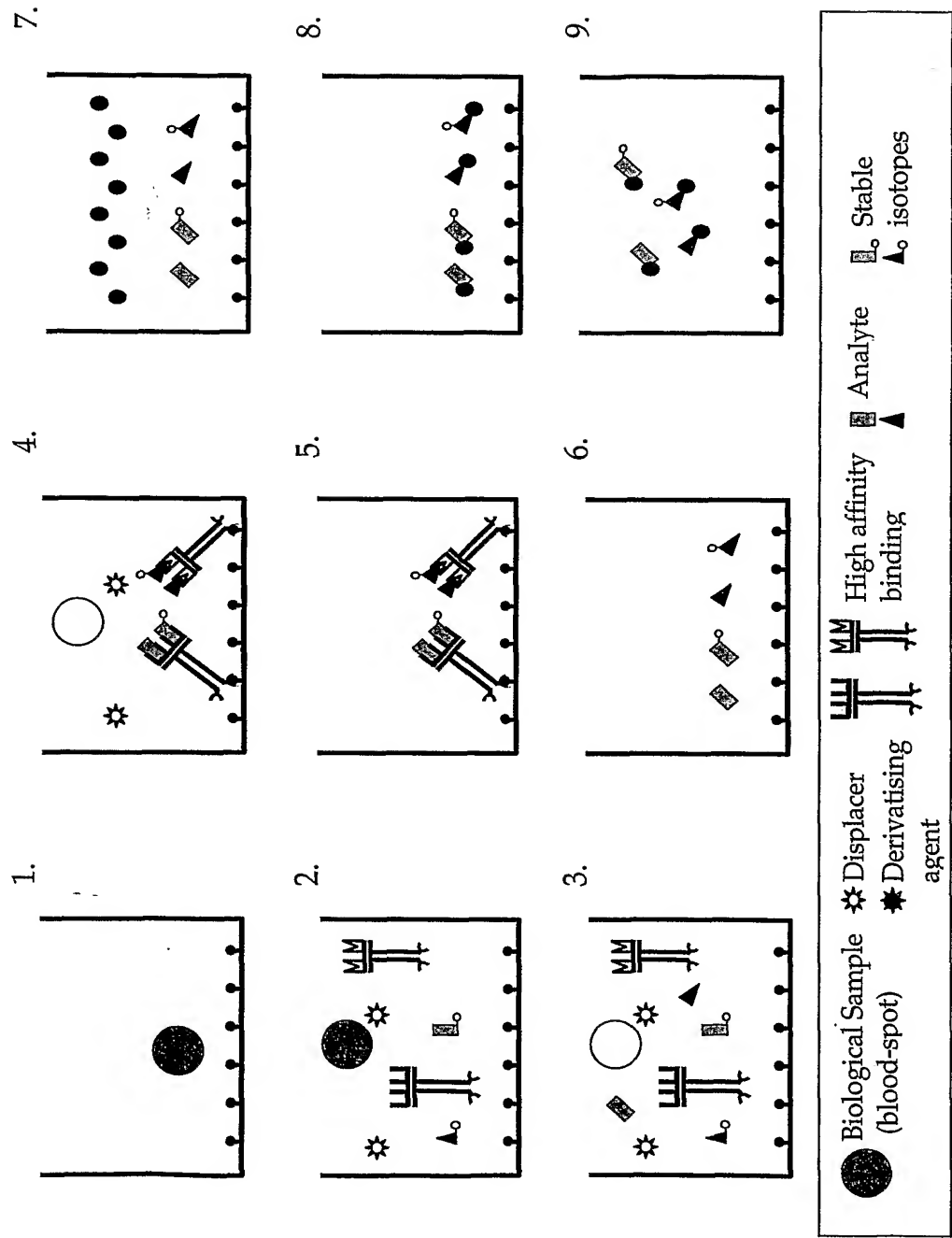
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of measuring the quantity of an analyte in a biological sample taken from a mammal, including the following steps:
 - (a) preparing at least one biological sample, in which said analyte is present, from said mammal;
 - (b) capturing said analyte from said sample, using an agent having affinity for said analyte;
 - (c) coupling one or more detectable moieties to said analyte to enhance detection and quantification; and
 - (d) quantifying the levels of said analyte by mass spectrometry.
2. A method according to claim 1, wherein the said analyte has a mass to charge ratio (m/z) of up to 2000.
3. A method according to claim 1 or 2, wherein said analyte is thyroxine or 17- α -hydroxy-progesterone, or a derivative thereof.
4. A method according to any one of claim 1, wherein the method is adapted to detect said thyroxine at concentrations up to 1 μ M in said biological sample.
5. A method according to any one of claims 1 to 4, wherein said agent having affinity for said analyte is selected from the group consisting of globulin proteins, monoclonal or polyclonal antibodies, sera, aptomers and antibody or receptor fragments; or derivatives thereof.
6. A method according to claims 1 to 4 wherein said agent having affinity for said analyte are monoclonal antibodies with affinity for T₄ or 17OHP.

7. A method according to any one of claims 1 to 6, wherein said mammal is a newborn human.
8. A method according to claim 7, for the mass screening of newborns.
9. A method according to any one of claims 1 to 8, for screening for congenital hypothyroidism or congenital adrenal hyperplasia.
10. A method according to any one of claims 1 to 9, wherein said detectable moiety is in the form of an acyl ester.
11. A method according to claim 10, wherein said detectable moiety is a butyl ester
12. A method according to claim 11, wherein said detectable moiety is a butyl ester and the said analyte is thyroxine.
13. A test kit to be used for the detection and/or determination of the concentration of an analyte including:
 - (a) microtitre plates containing wells that are coated with avidin
 - (b) additional immuno chemical reagents including:
 - (i) an elution buffer containing a displacer of salicylate
 - (ii) an aqueous buffer containing agent(s) coupled with biotin molecules and having affinity for said analyte
 - (iii) a solution having stable isotopes of T₄ and 170HP
 - (iv) a solution having a chemical derivatisation reagent
 - (v) a washing buffer
 - (vi) a solvent delivery buffer

14. A test kit according to claim 13 wherein said agent(s) coupled with biotin molecules are in the form of one or more of the following, monoclonal or polyclonal antibodies, globulin proteins, sera, aptomers and antibody or receptor fragments or derivatives thereof.
15. A test kit according to any of the claims 13 or 14 wherein said chemical derivatisation agent results in the butylation of the said analyte.
16. A test kit according to any of the claims 13 to 15, wherein said analyte is T₄ or 170HP.

Fig 1 A diagrammatic representation of the steps in the SMAC-MS process



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Fig 2

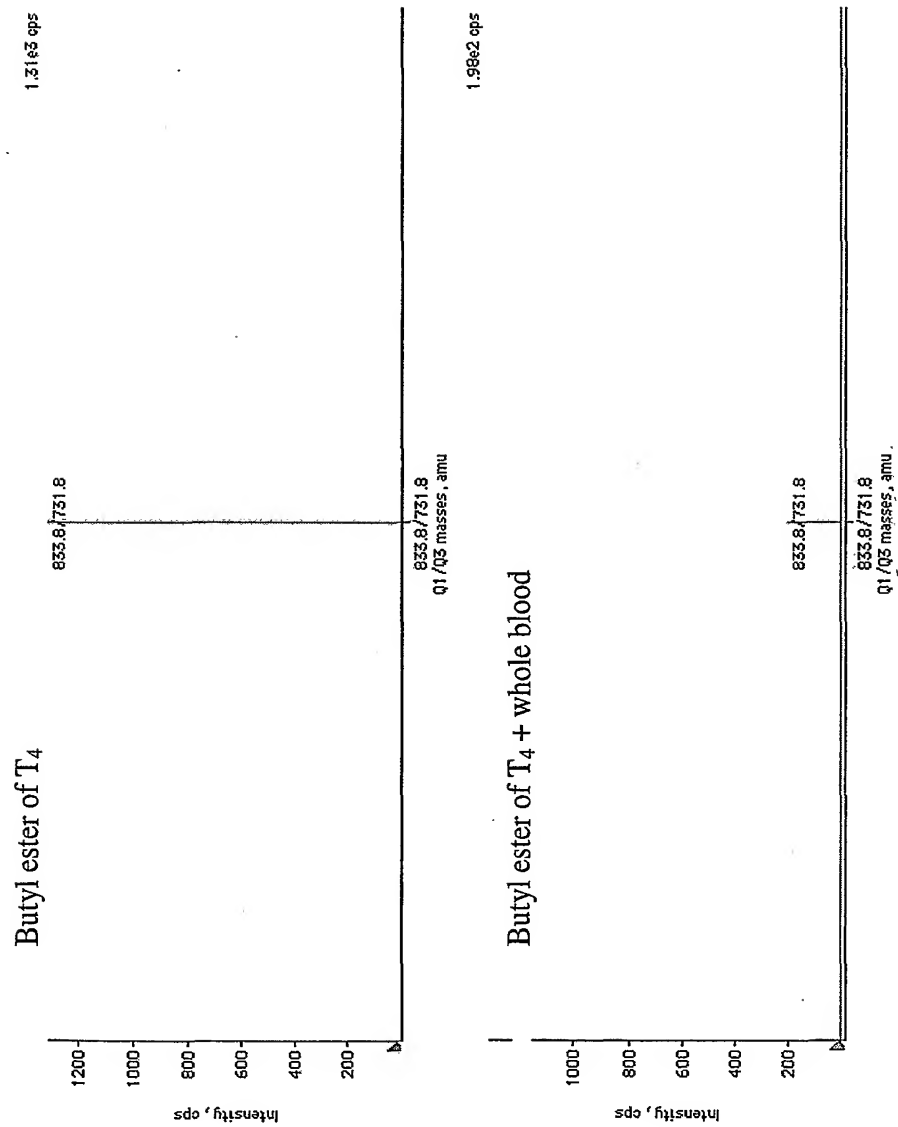


Fig 3

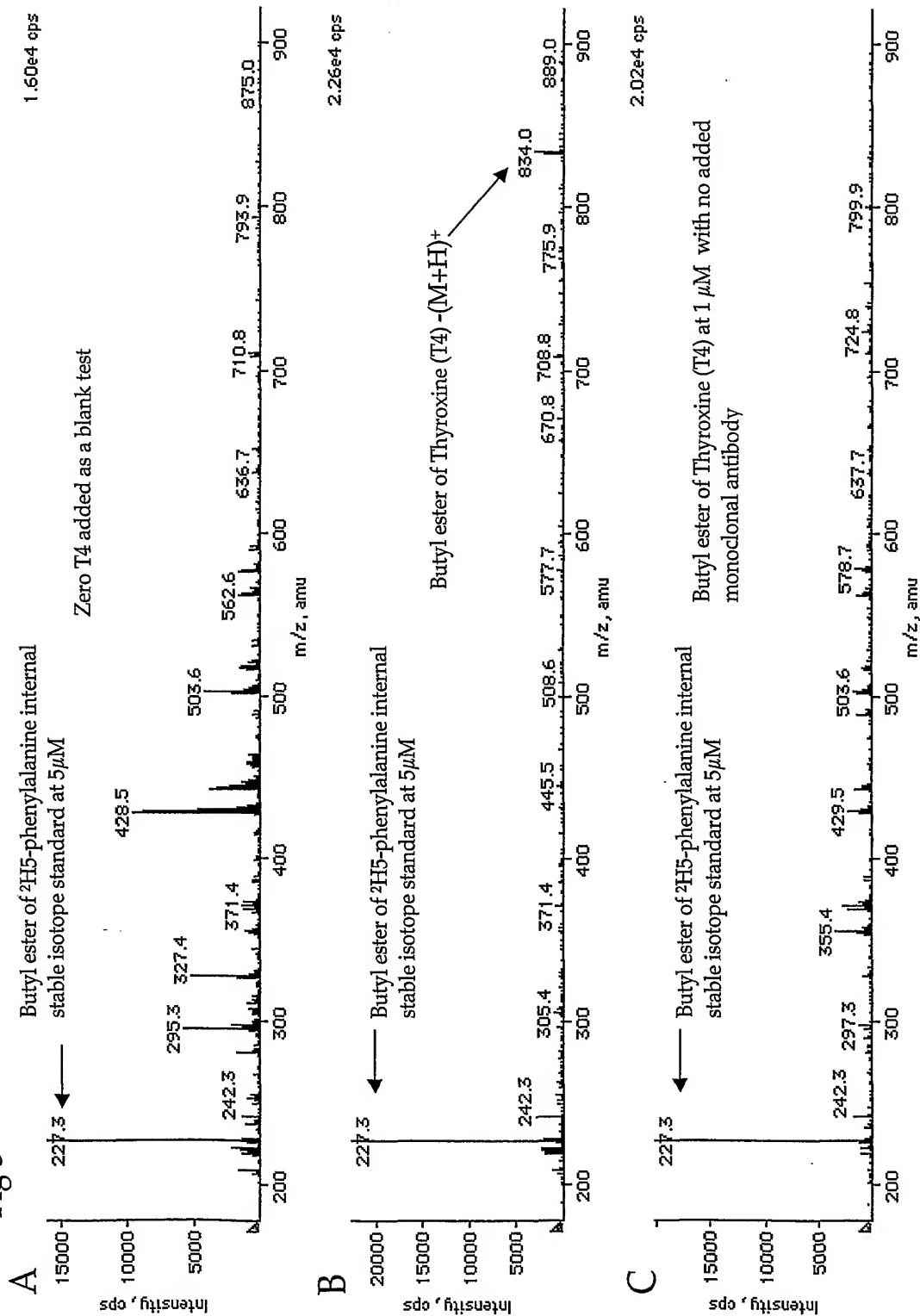
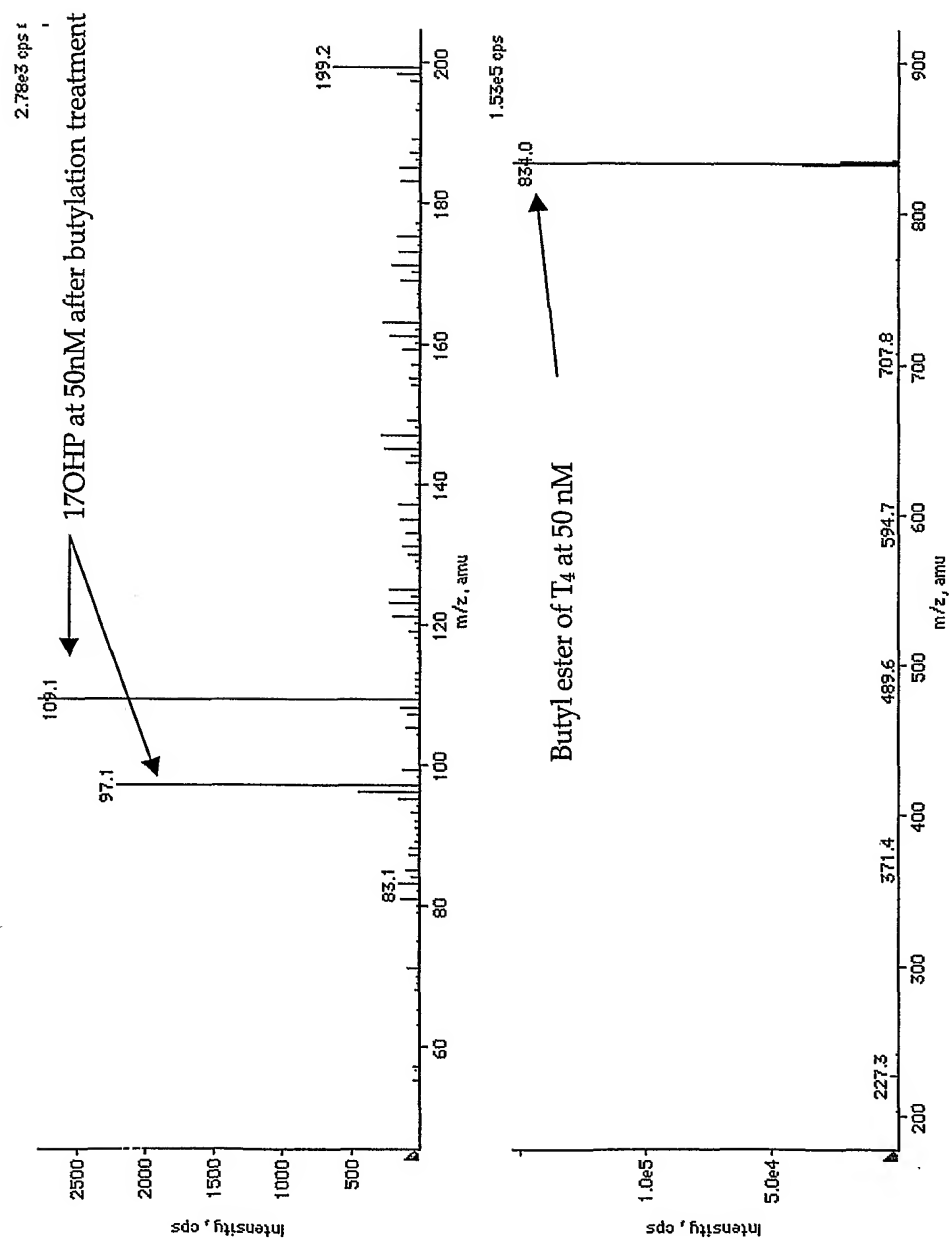


Fig 4



INTERNATIONAL SEARCH REPORT*

International application No.

PCT/AU01/01583

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl. ⁷: G01N 33/78, 33/74, 33/49, 30/72

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl. ⁷: Refer electronic databases consulted

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DWPI G01N 33/78, 33/74, 33/49, 30/72 and keywords (thyroxine, mass spectrometry, progesterone, hydroxyprogesterone, neonatal and derivatives) esp@cenet, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents:			
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

8 February 2002

Date of mailing of the international search report

18 FEB 2002

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/01583

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos : 1, 2
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims to the measurement of an "analyte", use of an "agent having affinity for said analyte" and use of "detectable moieties" are broad terms for which the specification does not provide support. The discussion of the proposed invention, see for example pages 11 to 13, is related to very specific "analytes", "agents", and "detectable moieties". The terms of claims 1 and 2 are so broad that they do not allow a meaningful search to be carried out.
3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.